Published in final edited form as:

Neuroreport. 2006 November 27; 17(17): 1787–1791.

Response of matrix metalloproteinase-9 to olfactory nerve injury

Richard M. Costanzo^a, Lisa A. Perrino^a, and Masayoshi Kobayashi^{a,b}

^aDepartment of Physiology, School of Medicine, Virginia Commonwealth University, Richmond, Virginia, USA

^bDepartment of Otorhinolaryngology Head and Neck Surgery, Mie University Graduate School of Medicine, Tsu, Mie, Japan

Abstract

Matrix metalloproteinases function in the remodeling of the extracellular matrix during growth and development as well as in injury and disease processes. We examined the role of matrix metalloproteinase-9 in a model of olfactory nerve injury in mice. We measured changes in matrix metalloproteinase-9 protein levels for up to 60 days following olfactory nerve transection. Matrix metalloproteinase-9 levels increased within hours after injury, peaked at day 1 and were elevated for approximately 2 weeks before returning to control levels over the 60-day time period. The increase in matrix metalloproteinase-9 was temporally associated with the degeneration of olfactory neurons that follows nerve transection and with increased gliosis. Our results demonstrate a temporal relationship between matrix metalloproteinase-9 elevation, degeneration of olfactory neurons and gliosis.

Keywords

central nervous system; degeneration; glial fibrillary acidic protein; matrix metalloproteinase-9; nerve injury; olfactory bulb

Introduction

Matrix metalloproteinases (MMPs) are a family of proteinases with the ability to degrade components of the extracellular matrix (ECM). MMPs play crucial roles in normal extracellular remodeling and development, as well as in recovery from injury and in reparative processes. Owing to their powerful proteolytic ability, MMP activity can also be involved in pathological conditions such as the spread of metastatic cancer, and neurodegenerative processes [1,2], as well as in neuronal injury and inflammation [3,4]. A subclass of MMPs, the type IV collagenases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are frequently found in the central nervous system (CNS) and they have the ability to degrade components of the ECM and vascular basement membranes. Differences in the temporal expression of these two gelatinases have been reported [4–8]. MMP-9 is often elevated immediately after injury, particularly during the onset of inflammation, although its role is not yet clear [4,9,10]. MMP-2 is often constitutively expressed [11] or expressed after the initial response to injury [12]. Recent studies of injury in the CNS have implicated rapid MMP-9 upregulation in early stages following acute CNS injury, including traumatic brain injury [10], ischemia [5,6,8,13] and spinal cord injury [9].

In this study, we used the olfactory system as an injury model. The olfactory system provides a unique model for studying neuronal injury and repair because, unlike other neurons in the CNS, olfactory neurons are continuously replaced over the entire lifespan and have a remarkable capacity for neurogenesis. Olfactory neurons are also replaced in response to injury and retain the ability to reestablish connections with the olfactory bulb. Previous studies have established a well defined time course and level of recovery following injury to the olfactory nerves [14,15]. During the degeneration phase that occurs over the first several days following nerve injury, sensory neurons degenerate whereas basal cells in the epithelium proliferate. This is followed by a regeneration phase in which new cells in the epithelium grow axons to the olfactory bulb and reestablish connections between the epithelium and the olfactory bulb.

Although this time course of events following injury is well defined, little is known about the regulation of the responses and mechanisms that mediate events beginning with initial injury through the degeneration, regeneration and recovery processes. Therefore, we explored the potential association of MMP-9 with some of these processes, specifically with degeneration and gliosis. Given MMP-9's documented modulation in the CNS and the unique capability of the olfactory system to serve as an injury model, we hypothesized that MMP-9 would be modulated in the olfactory bulb in response to olfactory nerve injury.

Methods

Surgical procedures

Adult P2-IRES-tau-lacZ mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) and olfactory nerve transections were performed as previously described [16]. After anesthesia, bilateral olfactory nerve transection was achieved by exposure of the olfactory bulbs and by insertion of a thin, Teflon cutting blade between each olfactory bulb and the cribriform plate (Fig. 1a). The use of a Teflon blade resulted in transection of all the olfactory axons that connect to the olfactory bulbs, and minimal damage to the olfactory bulbs and cribriform plate. After transection, the skin incision was sutured and each animal was observed postoperatively before returning to its cage. Sham surgeries were also performed to control overall effects of anesthesia and surgical exposure of the two olfactory bulbs. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Tissue sampling and preparation

At 5 h and 1, 3, 7, 10, 15, 35 and 60 days after the bilateral olfactory nerve transection, mice were anesthetized with sodium pentobarbital and killed by rapid decapitation, and olfactory bulb tissue was removed. Olfactory bulb tissue from control animals (receiving no surgical treatment) and sham animals was obtained using the same procedure. The bulbs were removed from the skull, and the anterior-ventral portions (containing the injured surface) were removed for protein measurement (Fig. 1b). Samples from the left and right bulbs were pooled and immediately placed in protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium dodecylsulfate, 1% DOC) containing a protease inhibitor cocktail (Calbiochem, San Diego, California, USA), and homogenized with a motor-driven plastic homogenizer. The tissue was incubated on a rotating platform for 20 min at 4°C and centrifuged at 16 000g for 30 min at 4°C. Solubilized proteins in the supernatant were quantified with a DC protein assay reagent kit (Bio-Rad Laboratories, Hercules, California, USA), using bovine serum albumin as a standard. Protein measurements were made at 720 nm on a UQuant plate reader (BioTek Instruments Inc., Winooski, Vermont, USA). Equal protein samples and purified murine MMP-9 (Chemicon, San Francisco, California, USA) were loaded onto Bis-Tris 4–12% density gradient gels and separated using NuPAGE MES [2-(*N*-morpholino) ethane sulfonic acid] reducing buffer system (Invitrogen, Carlsbad, California, USA) for 1 h at 200 V and 4°C.

C. Protein was transferred to nitrocellulose membranes for 2 h at 25 V and 4°C. Nonspecific binding was blocked with 5% bovine, nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 for 1 h. Primary antibodies against MMP-9 (AF909, 1 : 200) were obtained from R&D Systems (Minneapolis, Minnesota, USA). Olfactory marker protein (OMP) (1 : 20 000; Wako Chemicals, Richmond, Virginia, USA) and glial fibrillary acidic protein (GFAP) (1 : 50 000; Dako North America, Carpinteria, California, USA) were used as markers of olfactory degeneration and injury. Anti-cyclophilin A (CPA) (1 : 5000; Upstate, Lake Placid, New York, USA) antibody was used to standardize protein loading. Nitrocellulose membranes were incubated overnight in primary antibody at 4°C. Membranes were then treated with species-specific peroxidase-conjugated IgG (Rockland, Gilbertsville, Pennsylvania, USA), incubated for 1 min with Western Lightning Plus reagent (Perkin Elmer, Wellesley, Massachusetts, USA) and exposed to Blue Sensitive Autoradiography film (Marsh BioProducts, Rochester, New York, USA).

Protein measurement

Quantification of protein expression was performed using Quantity One Analysis software (Bio-Rad Laboratories). Protein expression was measured as a function of both band density and band area. This density–area measurement for MMP-9, OMP and GFAP at each recovery time point was then standardized against the corresponding density–area measurement for CPA in the same gel. The protein measurement of MMP-9, OMP or GFAP was then expressed as a ratio of CPA (amount of protein divided by amount of cyclophilin A).

Results

After olfactory nerve transection, MMP-9 protein expression was measured in the olfactory bulb shortly after the injury and during neuronal degeneration and regeneration. Western blot analysis of the time course of MMP-9 expression is shown in Fig. 2. Density–area measurements of the MMP-9 protein bands were compared with that of CPA (Fig. 2a) to calculate the relative amounts of protein at each recovery time point. Relative amounts of MMP-9 from two separate experiments are plotted in Fig. 2b. MMP-9 protein was detectable as early as 5 h following injury and levels increased approximately two to three-fold within a 24-h time period. Although MMP-9 levels remained elevated above control for about 2 weeks after injury, a decline toward control levels was observed over the remaining time period (60 days). Olfactory bulb samples from sham animals measured at recovery days 1 and 3 had no detectable levels of MMP-9 (data not shown).

In addition to the changes in MMP-9 following olfactory nerve injury, markers associated with neuronal degeneration and gliosis were measured using Western blot analysis (Fig. 3a). OMP, expressed only by mature olfactory neurons with established connections to the olfactory bulb, allowed us to assess the time course of degeneration following olfactory nerve injury. Relative amounts of OMP are plotted in Fig. 3b. The increase in MMP-9 expression was temporally associated with the degeneration of olfactory nerves, measured by changes in OMP expression following olfactory nerve transection. OMP levels in the olfactory bulb decreased and remained well below control levels during the degeneration period, several days following nerve transection. OMP levels returned to control levels during regeneration. Specifically, OMP levels dropped to almost one-half of control levels between day 1 and day 3, when MMP-9 levels were substantially elevated. OMP remained very low through day 10 and recovered to control levels between day 10 and day 15. These changes reflect the established time course of degeneration and regeneration of olfactory neurons that follow injury. During maximum degeneration (days 3–10), MMP-9 levels were elevated above control levels. At the onset of regeneration (day 10), MMP-9 levels began to decline.

Measurement of GFAP levels allowed us to assess the degree of gliosis that develops after CNS injury (Fig. 3a). GFAP is constitutively produced by astrocytes. In the reactive glial response to CNS injury, however, hypertrophic, reactive astrocytes increase their expression of the intermediate filament protein, GFAP [17]. Relative amounts of GFAP following olfactory nerve transection are plotted in Fig. 3b. The increase in MMP-9 was temporally associated with significant astrocyte activation, measured by the GFAP increase. After olfactory nerve transection, GFAP levels in the bulb rose significantly several days following injury and increased steadily before reaching a plateau. The GFAP increase began at day 3 when MMP-9 levels were still elevated. The onset of GFAP elevation at day 3 also corresponded to the OMP decrease. GFAP increased sharply after day 3, to levels two-fold higher than control levels. GFAP levels reached a maximum at day 10 and remained elevated for the remainder of the 60-day time course. The GFAP peak at day 10 corresponded with the onset of OMP recovery.

Discussion

MMP-9 has been the focus of recent studies in the CNS where its marked elevation after injury is correlated with early injury processes and deleterious effects such as inflammation, degradation of the blood–brain barrier (BBB), edema and neuronal death. As observed in our study of the olfactory bulb, the MMP-9 increase reported for other CNS injuries [6,10,18,19] was also relatively short-lived, peaked within 1–3 days and then declined over the course of several days to weeks [5,7,9]. MMP-9's return to control levels within several weeks suggests that MMP-9 does not play a major role at later time points in the recovery process.

Rapid onset, reactive processes such as inflammation, gliosis and neuronal degeneration found in CNS injuries typically occur within hours to several days after injury [20] and coincide with the initial MMP-9 elevation following olfactory nerve injury. Our observation that MMP-9 increased within 5 h of olfactory nerve injury suggests a strong correlation to inflammatory processes such as microglia activation and infiltration of leukocytes. The rapid onset and peak of MMP-9 in the olfactory bulb is consistent with the timing of the breakdown of the BBB observed in other CNS injuries when vascular integrity begins to diminish [13]. MMP-9 activity in endothelial cells within cerebral vasculature around damaged tissue may contribute to mechanisms that, in part, lead to edema and leukocyte infiltration [5,21]. MMP-9 is capable of digesting matrix proteins present in vascular basal lamina such as collagen, fibronectin and laminin, providing a pathway for destruction of vascular integrity in damaged tissue. The early migration of neutrophils provide a source of MMP-9 following acute injury [5,21,22]. Our results, together with other CNS injury studies, suggest that the early MMP-9 increase after injury may be associated with an early vascular response.

The temporal correlation observed between the onset of MMP-9 elevation and gliosis, indicated by the significant increase in GFAP, suggests that MMP-9 may play a role in the process of glial scar formation. We observed that GFAP increased 3 days following olfactory nerve injury, and that this coincided with the typical onset of reactive gliosis following CNS injury [19]. GFAP continued to increase until day 10, when its expression leveled off and was maintained above control levels, presumably indicating a period of glial scar maturation. MMP-9 levels also remained elevated over this same time period. At 1–2 weeks after injury, MMP-9 levels began to decrease, whereas GFAP expression remained high. The formation of the glial scar relies on the introduction of vascular components into CNS tissue achieved through BBB disruption. This disruption is present for 1–2 weeks following CNS injury and coincides with the observed elevation of MMP-9 following olfactory nerve injury. Although astrocyte activation and the initiation of glial scarring (marked by GFAP expression) are initially dependent on the disruption of the BBB, glial scar maturation may subsequently contribute to the repair of the BBB over the course of several weeks [23]. One possible interpretation for

our data is that the early MMP-9 elevation and its correlation with the GFAP increase are the result of MMP-9's disruption of the BBB, leading to the initiation of gliosis and the formation of the glial scar. The subsequent plateau of GFAP and the decrease of MMP-9 levels may be a signal of the maturation of glial scar that helps to restore BBB integrity [17].

Using the olfactory system as the injury model also enabled us to study the timing of neuronal degeneration and its relationship to changes in MMP-9. Degeneration of olfactory neurons typically occurs over the course of several days following injury, and although the time frame of injury and degeneration has been established, the mechanisms that promote the degeneration are not well understood. Owing to the gaps in our understanding of the mechanisms regulating degeneration and regeneration, the potential role of MMP-9 in response to olfactory nerve injury is interesting. Our data suggest that following olfactory nerve transection, MMP-9 may play a role in degeneration. MMP-9 levels remained elevated above control levels over the course of olfactory neuron degeneration as illustrated by the OMP decrease from day 3 to day 10. OMP began to return to control levels after 10 days and this decrease coincided with the decrease in MMP-9. One hypothesis for the role of MMP-9 in neural degeneration is that MMP-9 induces neuronal death by disruption of the neuron–ECM interactions [6,24]. In CNS injuries in which there is no BBB disruption, MMP-9 has been primarily localized to neurons [6,25], supporting the idea that MMP-9 could play a role in neural degeneration.

Conclusion

This is the first report demonstrating that MMP-9 is associated with events following olfactory nerve injury. The results show a temporal relationship between MMP-9, neuronal degeneration and gliosis following olfactory nerve injury. Further investigation using the olfactory nerve injury model may provide a better understanding of how MMP-9 contributes to the underlying mechanisms regulating injury and recovery processes. The modulation of MMP-9 may prove to be important in the development of new treatment strategies, to improve outcome and recovery following injury in the CNS.

Acknowledgements

We thank Dr Helen Fillmore for technical advice and comments during the preparation of the manuscript.

Sponsorship: This work was supported by Grant DC000165 from the National Institute on Deafness and Other Communication Disorders.

References

1. Hartung HP, Kieseier BC. The role of matrix metalloproteinases in autoimmune damage to the central and peripheral nervous system. *J Neuroimmunol* 2000;107:140–147. [PubMed: 10854648]
2. Dzwonek J, Rylski M, Kaczmarek L. Matrix metalloproteinases and their endogenous inhibitors in neuronal physiology of the adult brain. *FEBS Lett* 2004;567:129–135. [PubMed: 15165905]
3. Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. *Nat Rev Neurosci* 2001;2:502–511. [PubMed: 11433375]
4. Rosenberg GA. Matrix metalloproteinases in neuroinflammation. *Glia* 2002;39:279–291. [PubMed: 12203394]
5. Romanic AM, White RF, Arleth AJ, Ohlstein EH, Barone FC. Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. *Stroke* 1998;29:1020–1030. [PubMed: 9596253]
6. Lee SR, Tsuji K, Lee SR, Lo EH. Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. *J Neurosci* 2004;24:671–678. [PubMed: 14736853]
7. Goussev S, Hsu JY, Lin Y, Tjoa T, Maida N, Werb Z, et al. Differential temporal expression of matrix metalloproteinases after spinal cord injury: relationship to revascularization and wound healing. *J Neurosurg Spine* 2003;99:188–197.

8. Planas AM, Sole S, Justicia C. Expression and activation of matrix metalloproteinase-2 and -9 in rat brain after transient focal cerebral ischemia. *Neurobiol Dis* 2001;8:834–846. [PubMed: 11592852]
9. Noble LJ, Donovan F, Igarashi T, Goussev S, Werb Z. Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J Neurosci* 2002;22:7526–7535. [PubMed: 12196576]
10. Wang X, Jung J, Asahi M, Chwang W, Russo L, Moskowitz MA, et al. Effects of matrix metalloproteinase-9 gene knock-out on morphological and motor outcomes after traumatic brain injury. *J Neurosci* 2000;20:7037–7042. [PubMed: 10995849]
11. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;17:463–516. [PubMed: 11687497]
12. Tsukatani T, Fillmore HL, Hamilton HR, Holbrook EH, Costanzo RM. Matrix metalloproteinase expression in the olfactory epithelium. *Neuroreport* 2003;14:1135–1140. [PubMed: 12821796]
13. Fujimura M, Gasche Y, Morita-Fujimura Y, Massengale J, Kawase M, Chan PH. Early appearance of activated matrix metalloproteinase-9 and blood–brain barrier disruption in mice after focal cerebral ischemia and reperfusion. *Brain Res* 1999;842:92–100. [PubMed: 10526099]
14. Costanzo RM. Neural regeneration and functional reconnection following olfactory nerve transection in hamster. *Brain Res* 1985;361, 258–266.
15. Schwob JE. Neural regeneration and the peripheral olfactory system. *Anat Rec* 2002;269:33–49. [PubMed: 11891623]
16. Yee KK, Costanzo RM. Restoration of olfactory mediated behavior after olfactory bulb deafferentation. *Physiol Behav* 1995;58:959–968. [PubMed: 8577894]
17. Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004;5:146–156. [PubMed: 14735117]
18. Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, et al. Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood–brain barrier and white matter components after cerebral ischemia. *J Neurosci* 2001;21:7724–7732. [PubMed: 11567062]
19. Duchossoy Y, Horvat JC, Stettler O. MMP-related gelatinase activity is strongly induced in scar tissue of injured adult spinal cord and forms pathways for ingrowing neurites. *Mol Cell Neurosci* 2001;17:945–956. [PubMed: 11414785]
20. Berry, M.; Butt, A.; Logan, A. Cellular responses to penetrating CNS injury.. In: Berry, M.; Logan, A., editors. *CNS injuries: cellular responses and pharmacological strategies*. CRC Press; Boca Raton, Florida: 2000. p. 1-168.
21. Rosenberg GA, Cunningham LA, Wallace J, Alexander S, Estrada EY, Grossetete M, et al. Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain Res* 2001;893:104–112. [PubMed: 11222998]
22. Lo EH, Wang X, Cuzner ML. Extracellular proteolysis in brain injury and inflammation: role for plasminogen activators and matrix metalloproteinases. *J Neurosci Res* 2002;69:1–9. [PubMed: 12111810]
23. Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, et al. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 1999;23:297–308. [PubMed: 10399936]
24. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, et al. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 2002;297:1186–1190. [PubMed: 12183632]
25. Szklarczyk A, Lapinska J, Rylski M, McKay RD, Kaczmarek L. Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. *J Neurosci* 2002;22:920–930. [PubMed: 11826121]

Fig. 1.

Diagram illustrating the olfactory nerve transection procedure and location of tissue sampled.

(a) Insertion of a Teflon blade between the olfactory bulbs and the cribriform plate results in the complete transection of the olfactory nerves passing from the nasal cavities to the olfactory bulbs. (b) At different time points in recovery, the right and left olfactory bulbs were removed from the skull, cut (along dotted line) and the anterior–ventral halves pooled for protein measurement in Western blot.

Fig. 2.

Analysis of matrix metalloproteinase (MMP)-9 protein expression in the olfactory bulb following olfactory nerve transection injury. (a) Representative Western blot (experiment 1) showing changes in MMP-9 at different expression time points following injury. Lane 1 shows purified murine MMP-9 standard. Lane 2 shows the absence of MMP-9 in control tissue (CTRL). Cyclophilin A (CPA) bands in each lane were used to adjust for differences in protein loading. (b) Plot of the relative amount of MMP-9 (band density–area values) from two separate experiments expressed as a percentage of (CPA). Each experiment represents data obtained from different mice. MMP-9 was detected at the 5-h time point. MMP-9 levels peaked at day 1, were elevated until day 15, and then declined, reaching control levels by day 60.

Fig. 3. Analysis of olfactory marker protein (OMP) and glial fibrillary acidic protein (GFAP) expression in the olfactory bulb following olfactory nerve transection injury. (a) Representative Western blot (experiment 1) showing changes in OMP and GFAP levels at different time points following injury. Bands in the control lanes (CTRL) represent measurements from control animals. Cyclophilin A (CPA) bands in each lane were used to adjust for differences in protein loading. (b) Plot of the relative amount of protein (band density-area values) for OMP and GFAP expressed as a percentage of CPA. Three days after injury a decrease in OMP was observed, representing the degeneration of olfactory nerves. In addition, GFAP, representing astrocytic activation, increased during the same time period as neural degeneration. The changes in both OMP and GFAP correspond temporally to the changes in MMP-9 levels.